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In re: Walsh et al.
Appl. No. 09/689,430
Filed October 12, 2000

APPENDIX A

3. contest of F dy in day 1 supernatants of ~~rAA~~ pdLZ2 infected 3T3 & HepG2 cells

TABLE OF ABSORBANCE VALUES

FILE: FILE 1 TITLE: FILE 1 REPORT DATE AT 05:40 PM

		1	2	3	4	5	6	7	8	9	10	11	12
ddH2O →	A	0.066	0.020	-0.006	-0.060	-0.088	-0.099	-0.105	-0.108	-0.093	-0.103	-0.111	-0#110
		20	14.3	9.1	4.8	1.2	Blank	3T3/F8	3T3	DMZM	HepG2/F8	HepG2	MZM
duplicate	B	1.201	0.944	0.705	0.495	0.296	0.220	0.227	0.232	0.221	0.216	0#234	0#426
	C	1.025	0.916	0.660	0.495	0.305	0.245	0.073	0.296	0.247	0.224	0#213	0#224
Nothing →	D	-0.123	-0.120	-0.124	-0.121	-0.119	-0.119	-0.123	-0.124	-0.121	-0.122	-0.122	-0#124

4. Re-set ultra-spin of rAA/F8 viruses from 40 plates.

tube ④ ⑤. ⑥. ⑦. ⑧. → 2 x tubes

others → 2 x tubes.

41K. 15°C. X 60 h.

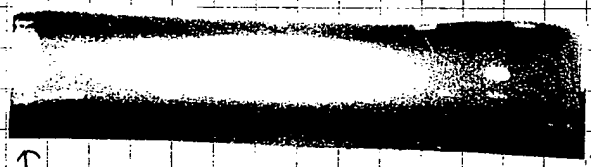
②. feed 3T3 & HepG2 / DLZ2 at 9:00 Am

⑤. PCR of Enhancer I of HBV. 2X

ddH2O	26 ul	template	10 ul (HBV/pUC19)
10 x Buffer	5 ul	PFu (Stratagene)	1 ul (2.5 u)
dNTPs	4 ul		50 ul
5'—P	2 ul (40 pmol)		
3'—P	2 ul (40 pmol)	mineral oil	50 ul

95°C 2 min
95°C 2 min
50°C 1 min | 8 cycles.
72°C 3 min
95°C 2 min
57°C 1 min | 20 cycles. → 72°C X 10 min
72°C 3 min
↓
4°C

loading PCR product → 2% agarose gel.



↑ 50 bp DNA ladder

100 bp from 50 bp system

Co-test of Day 2. 3. 4.

TABLE OF ABSORBANCE VALUES

FILE: TITLE: FILE 1
FACTOR: 1.000

	1	2	3	4	5	6
A	-0.002	-0.005	-0.003	0.001	-0.001	0.000
	20	14.3	9.1	4.3	1.4	0
double standard B	0.748	0.645	0.587	0.427	0.308	0.311
C	0.754	0.559	0.622	0.456	0.336	0.322
D	0.316	0.252	0.327	0.297	0.282	0.325
E	0.369	0.230	0.352	0.368	0.339	0.379
F	0.346	0.268	0.350	0.340	0.331	0.358
G	0.337	0.262	0.334	0.344	0.339	0.356

No f g ?

Day 5. 6 Sample

TABLE OF ABSURBANCE VALUES

FILE: TITLE: FILE 1 DATE: AT

FACTOR: 1.000

	1	2	3	4	5	6	7	8	9	10
	0	1.4	4.3	9.1	14.3	20?				
double standard A	0.265	0.355	0.470	0.648	0.847	0.993	0.001	~~0.004~~	0.001	-0.003
B	0.285	0.351	0.487	0.702	0.935	1.201	0.~~016~~	~~0.002~~	~~0.015~~	~~0.002~~
C	0.296	0.292	0.283	0.288	0.285	0.295	0.311	0.280	0.344	0.298
D	0.300	0.310	0.337	0.319	0.342	0.325	0.326	0.302	0.256	0.314

No f g

Sequency of TLC promoter is ok this ti

① Partial digestion of DLZ1-N3 with HindIII

from the previous experience, BglII can not cut DLZ2 well. so use relatively more HindIII for partial digestion

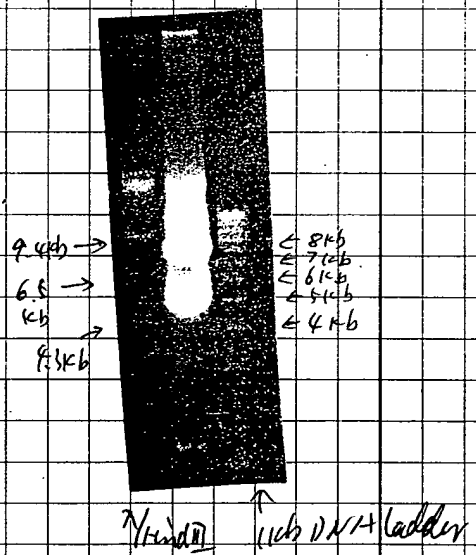
ddH2O	8 ul
10x reaction buffer 3	4 ul
DLZ1-N3	25 ul (8 ug)
HindIII	3 ul
	40 ul 37°C 1 hr

load to 0.7% agarose gel

cut the band between ~ 8 kb

purify through Qiagen gel kit

elute DNA in 100 uL 10mM Tris.Cl (pH8.5)



② purify EcoRI PCR products (100 ul) by QiaEX II kit

Elute DNA in 40 ul 10mM Tris (pH8.5)

③ EcoRI / BamH1 + HindIII

ddH2O
10× NewBamH1 Buffer 5.5
+BSA
EcoRI 40 ul
HindIII 2 ul
BamH1 2 ul

50 ul 37°C overnight

① Add 1 ul HindIII
1 ul BamH1
1.1 ul Buffer+BSA
6.9 ul ddH2O

37°C 4 h

② loading to 2% agarose gel.

③ purify the Bands by QiaEx II. elute DNA in 20 ul 10mM Tris-Cl (pH8.0)

④ Ligation

①. ddH2O 4 ul.

DLZ-/IIgIE + CLP 4 ul.

② DLZ-/IIgIE + CLP 6 ul

EnhI 2 ul.

③ DLZ-/IIgIE + CLP 7.5 ul

EnhI 1 ul

10x ligase buffer 1 ul

ligase 1 ul

(clontech)

16°C 18 h (overnight)

Transformation, 100 ul bacteria to each plate.

① 7 colonies.

② ≥200

③ ≥200 pick 20 colonies from ③

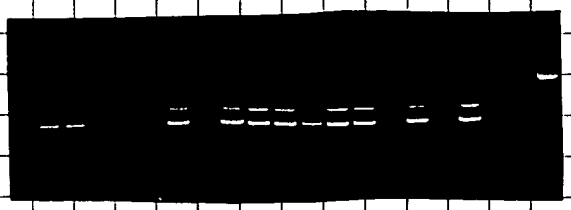
→ 3 ml LB/Amp 37°C overnight

① Extract DNA ② Digest with SmaI

① 1kb DNA ladder

②–⑳ : 1st~20th colony (two tiny) were lost

㉑ λ/HindIII



colony 1. 2. 7. 9. 10. 11. 12. 15. 17. 16. 18 have two ITRs.

cut 11 colonies with both ITRs with BglII + XhoI

ddH2O	2 μl
10×React 3	4
DNA	30
BglII	2
XhoI	2
	40 μl

37°C 2 h

① EcoRI right orientation between 5'ITR & TIC → 255/80

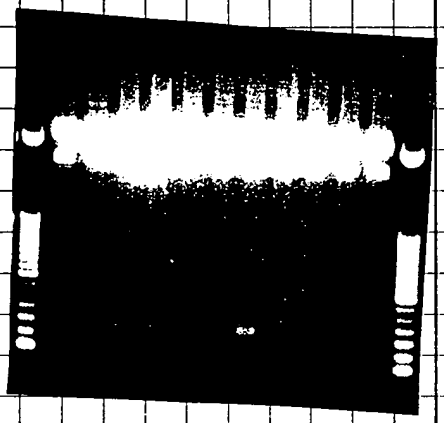
② ~~~~~ & P(A)n → 122/200

③ reverse orientation at 5' → 122/80

④ reverse orientation at 3' → 122/80

⑤

I can not see
250bp Band in any lane.



pick another 20 colonies from plate 2
→ 3ml LB/Amp 37°C overnight

Extract DNA, cut with BglII + XhoI
No 450bp band.

increase amount of EcoRI, set ligation again

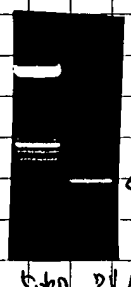
DNA / ~~ddH2O~~ + CIP	4 ul
EcoRI	4 ul
T4 ligase	1 ul
10x Buffer	1 ul

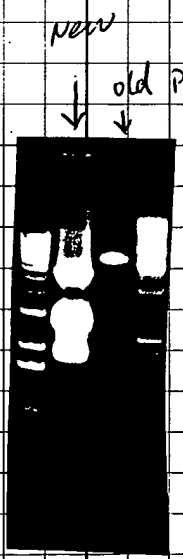
16°C 20 h

Transformati

The colonies on control (only pCZ2/MluI)
= pCZ2/MluI + EnhI
① forgot to do dephosphorylati

Reset pCZ2/BglII Partial Digest
ddH2O 8 μL
10× React } 4 μL
pCZ2-N3 25 μL
BglII 3 μL (Gibco)
40 μL 37°C 1 h
load to 0.7% agarose gel
Complete Digest !
Reduce the Enzyme to 1 μL
cut 7.5kbp Bands (vector)
1400bp Bands (inhancer)
elute vector in 40 μL
EnhI in 20 μL
in 1mM Tris-Cl (pH 8.0)





EcoRI vector

DNA 3 µl
dd H2O 2 µl
10x restriction buffer 5 µl
EcoRI 4 µl
(HF®)
50 µl 37°C 2 h

purify through QIAEX II kit
elute DNA in 20 µl 10 mM Tris pH

2. ligation

① Vector 4 µl
dd H2O 6 µl

② Vector 4 µl
insert 4 µl

③ Vector 6 µl
insert 2 µl

④ Vector 6 µl
insert 1 µl
dd H2O 1 µl

+ 1 µl ligase
1 µl 10x buffer
16°C overnight

Transformation?

① There are similar number of colonies (X-9/pcr) on ~~control~~ control and vector + insert plate

I do not know the reasons.

① poor-digested ends of the PCR-origin enhancer I ?

② The fragment of assumed DCE₂ are not the real one or the ?

Talk with Chris. I will start at very origin.

② Digest EcoR I PCR product with BamH I

10 ul from 20 ul of 9/1(P13) PCR product

ddH₂O 6.8 ul
10 ul DNA
10x NEBuffer BamH I 2.2 ul
+BSA
BamH I (NEB) 1 ul

20 ul 37°C overnight 15 h

Add ddH₂O 7.9 ul
10x NEBuffer+BSA 1.1 ul
BamH I 1 ul

/ 10 ul → 37°C 8 h

1. purify EnhI / BamH1 by QiaEX II kit elute DNA in 20 ul Tris-cl (10mM, pH 8.0).

2. ligati

EnhI / BamH1	4 ul.
TKtP(A) (MTE [illegible]) (3/20 Pχ2).	4 ul
T4 ligase (dm-tech)	1 ul.
10x ligase buffer	1 ul
	10 ul. 16°C 4 h.

3. PCR:

del H2O	26 ul	
dNTPs	4 ul	
10x buffer	5 ul	
A-P	2 ul	(3' primer of p(A)n)
S-P	2 ul	(5' primer of EnhI
template	10 ul	(ligati solion)
PFU	1 ul	
	50 ul	

mineral oil 50 ul.

95°C X ~.

95°C 2 min
50°C 1 min | 6 cycles
72°C 5 min

↓

95°C 2 min
57°C 1 min | 24 cycles
72°C 5 min

↓

72°C X15 min

↓

4°C

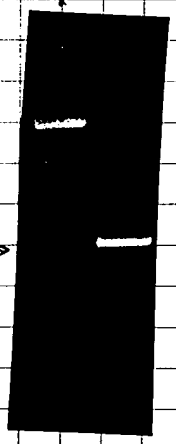
load the 2% agarose gel

cut the band at 350bp

purify by QIAEX II kit

elute in 40 μl X2 10mM tris-cl

20 μl 50bp DNA ladder (2 mg)



4. Test cut by XhoI

dd H2O	0
10×NEBuffer 2+BSA	4.4 μl
DNA	32 μl
XhoI	3 μl
	30 μl

37°C 2 h

5. by - BglII.

ddH2O	0
DNA	41 uL
10×React 3	5 uL
BglII	4 uL
	50 uL

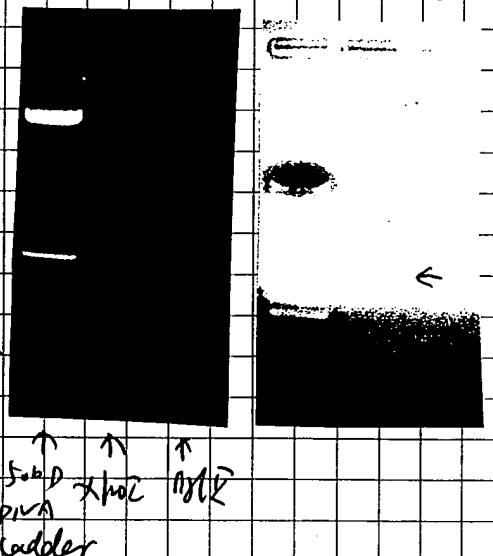
37°C overnight

① Add { ddH2O 8 uL; 10×React 3 1 uL; BglII 1 uL } → Ethanol / BglII

37°C 10 h

② load to 2% agarose gel

≈250 bp Band and 80 bp Band can be seen in XhoI lane



③ cut the BglII Band. purify through Qiagen kit. elute DNA in 20 uL 10 mM Tris-Cl

④ Ligation

② PTR/NdeI insert 6 ul (Page 66)

EcoRI+T4 PA 2 ul → PDL28

① PTR/NdeI+insert 4 ul

ddH2O 6 ul

1 ul T4 ligase + 1 ul 10x Buffer

16°C overnight

Transformation

① 14 colonies on plate 2

2 plate 1

Bad ligase?

pick 1 from plate to 3 ml LB/Amp

37°C roller-drum overnight

③. Ligation

4 ul EnhaI/BamH (P.103)

4 ul TK+MCS+PCA) (PAvay 1. from colony 4 & 7 of P(Z)3)

④. T4 ligase 1 ul (NEB) ← in notebook for making pPLZ4.

10x buffer 1 ul

16°C overnight

④. PCR
- ddH2O 26 ul
- dNTPs 4 ul
- A-P 2 ul (3' primer of P(A)n)
- S-P 2 ul (5' primer of EnhZ)
- template 10 ul (ligate system)
- PFU 1 ul

Total: 50 ul

95°C 2 min

95°C 2 min
50°C 1 min
72°C 5 min
6 cycles

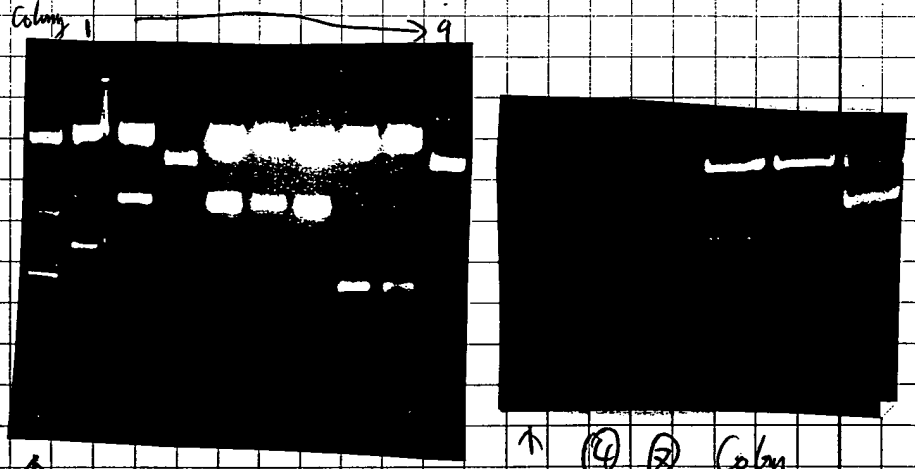
95° 1 min
57 1 min
72°C 5 min
24 cycle → 72°C X 15 min ↓ 4°C

② Extraction of plasmids pdt 7 & by Promega wizard mini-prep kit, elute DNA in 50 ul dd H2O.

③ Digest in with BglII

dd H2O	3 ul
10 x Peat 3	4 ul
DNA	30 ul
BglII	3 ul
	40 ul. 37°C 2 h.

load to 2% agarose gel



Colony 2. 8 seem ~~[illegible]~~ right insertion

cut PCR product of EcoRI + TK + MCS + P(A) (500bp)

elute DNA in 60 ul Tris-H (10 mM. pH 8.0)

(4) make more plasmids of colony 2 & 8.

① EnhTK+MCS+p(A) ⟶ ETMP. / BssHII

delH2O	3 ul
10×NewBuffer	
BssHII	4 ul
DNA	30 ul
BssHII	3 ul
	40 ul. 50°C 2 h

BglII

delH2O	11
10×React3	5
DNA	30
BglII	4
	50 ul

37°C overnight

② DLTS / XhoI

delH2O	0
10×NewBuffer3	5.5 ul
+BSA	
DNA	41 ul
XhoI	4 ul
	50 ul. 37°C 2 h

③ DLTS / SmaI

delH2O	10 ul
10×React3	3 ul
DNA	15 ul
SmaI	2 ul
	30 ul RT 2 h

load to 2% agarose gel

M : 50bp DNA Ladder

1 C7/SmaI

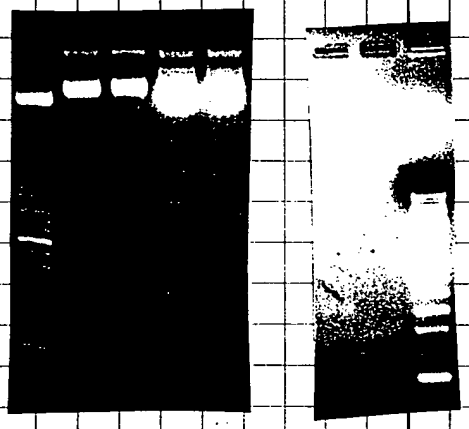
2 C8/SmaI

3 C7/XbaI

4 C8/XhoI

5 ETMP-4/BssHII

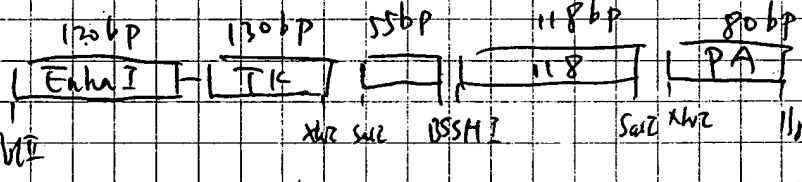
6 ETMP-7/BssHII



①: 0 at one ITR was deleted

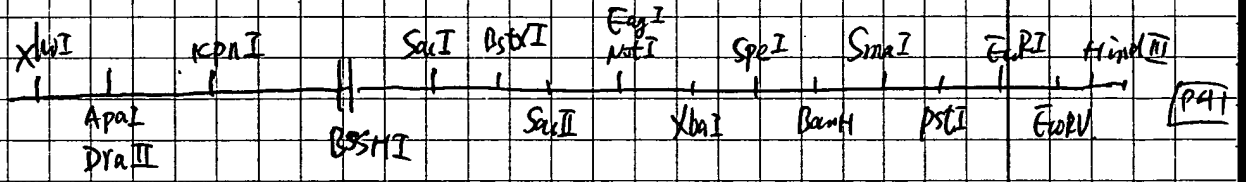
② seems no repeated insert of DC2-5 →

③ ETMP-4 : /BssHII ≒ 300 + 200

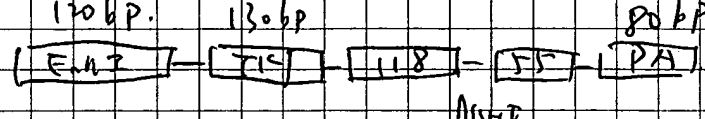


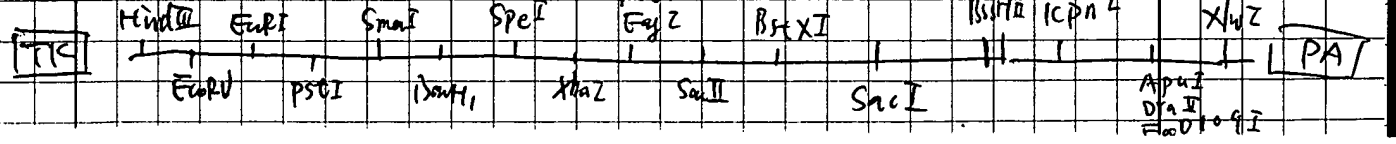
Results are comparable results of SnaBI digestion of TKTRSS tPA.

Rearrangement of MCS.



ETMP-7 ≒ 320 + 140





① Add BglII to ETMP/BglII system.

{ ddH₂O 16 ul
{ 10x React 3 2 ul
{ BglII 2 ul
20 ul 37°C. 6 h

② Extract DLZ5/XhoI from gel.
elute DNA in 50 X 2 10 mM Tris-Cl.

③. DLZ5/XhoI / BglII

DNA 41 ul
10x React 3 5 ul
BglII 4 ul
50 ul 37°C 2 h.

DLZ5 Colony 7 & 8
both OK

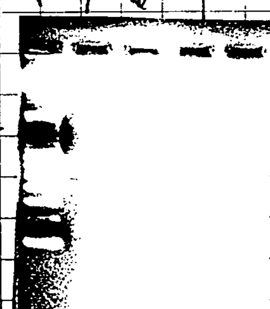
④ DLZ5/XhoI / CIP.

DNA 60 ul
10x NaMgDTT 3 8 ul
CIP 6 ul
ddH₂O 6 ul
80 ul 37°C 2 h

load to 2% agarose gel.
0.7%.

DLZ5 C7
XhoI/BglII
C8

500bp DNA ladder



ETMP-4
ETMP-

{ 4 ul 100 mM EDTA
{ 75°C X 10 min
{ purify by QIAEX
elute DNA in 20 ul TE
use colony 7 for next subcloning

⑨ pTR-EGFP from Rebecca.

swap EGFP replaced of GFP in pTR-UK1.

Digest with XbaI + BclI (BM) to get

EGFP + pA + TK + Neo ♦ cassette = 2265 bp.

vector = 4010.

ddH$_2$O	17 uL
10× Sure cut/H	4 uL
DNA	15 uL
BclI	2 uL
XbaI	2 uL
	40 uL 37°C 2 h

load ~~[redacted]~~ to 0.7% agarose gel.

Not cut by one enzyme:

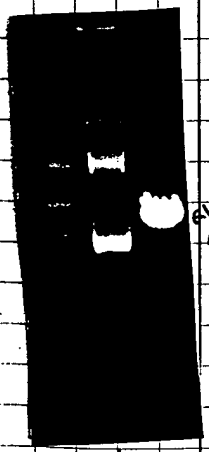
BclI ? or XbaI ?

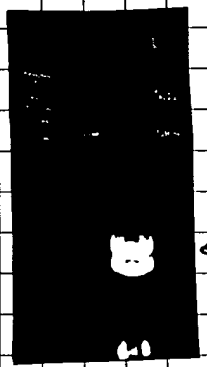
figure out which is bad.

ddH$_2$O	23 uL
pDC2	2 uL (1 ug)
XbaI (10× NuH+H/M	3 uL
BclI/XbaI	2 uL
	30 uL 37°C >2 h

Load to 0.7% agarose gel.

BclI is bad





⑧. CIP of pTR/BglII.

ddH2O

DNA 98 µl.

10×Buffer(NEB3) 12 µl.

CIP (NEB) 10 µl

120 µl. 37°C. 1 h.

6 µl 100mM EDTA 75°C × 10 min.

Desalt and concentrate by QiaEX II kit.

Elute DNA in 20 µl 10mM Tris-Cl (pH8.0)

⑨. Ligation

A. pCZ6-C7/XhoI+CIP 4 µl. AA and F8/XhoI

ddH2O 4 µl.

B. pCZ6-C7/XhoI+CIP 4 µl

F8/XhoI 4 µl.

C. pTR/BglII+CIP 3 µl

ddH2O 5 µl.

D. pTR/BglII+CIP 3 µl

EMP/4 5 µl

E. ETMP/7 just.

1 ml of ligase & 10 × buffer.

16°C overnight

Transformation. 100 ml cells/dish.

only 13 colonies on plate B

4 on D

2 on E

no colony in one-vol → Bad ligate? Control of transformation is ok.

pick colonies → 3 ml LB/Amp

37°C overnight

Repeat plating 200 ml cells on every dish

No improvement seen of colony number

Extract the plasmid by Promega Wizard miniprep kit elute DNA in 50 ml deH2O

Digestion.

① EcoRI + TIC + T8 + P(A) / XhoI.

ddH2o	14.7 ml
10 x NewBuffer 3	3.3
BSA	
DNA	10 ul
XhoI	2 ul
	30 ul. 37°C 2 h. (Overnight)

load to 0.7% agarose gel

① 1kb DNA ladder

②-⑬ Colony B1-12 / XhoI.

⑭ dfI / SmaI

② PTR + ~~EcoRI~~ ETMP / BglII

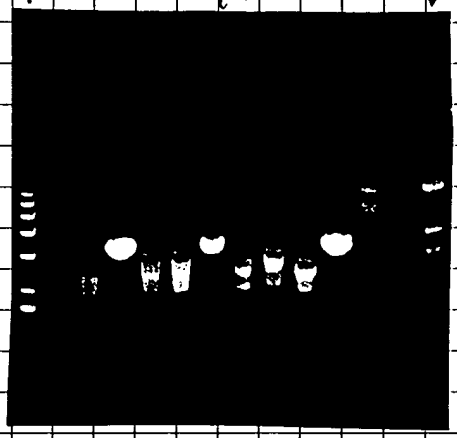
ddH2o	3 ul
10 x Buffer 3	25 ul
DNA	2 ul
BglII	
	30 ul

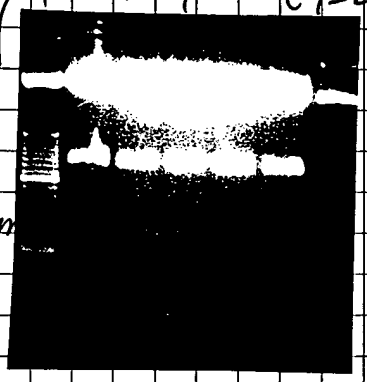
load to 2% agarose gel

① 50 bp DNA ladder.

②-⑤ Colony D1-4 (ETMP 4) 37°C overnight

⑥-⑦ Colony E1-2 (ETMP 2)

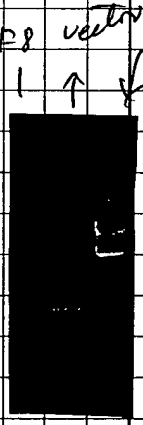




① No insert of F8 to vector

② ETMP4 has 4 insert

③ 7 1 insert



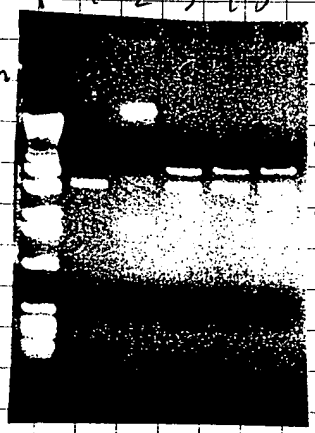
Load 5 ul of F8/XhoI & D(2,5)/C2
to 0.7% agarose gel

the failure of ligation perhaps is due to incorrect ratio between insert / vector

this time I will use ① { F8 = 6.0 uL, Vector = 2 uL ; ② { F8 = 5 uL, Vecto = 3 uL

for ligation

1. ran 10µl of 10µl ligat system on 0.7% agarose gel.



① vector / ctp.

② vector.

③ 4µl { vector / insert.

④ 3µl vector
5µl insert.

⑤ 2µl vector.
6µl insert

It seemed little ligat occurre even if there were some.
A little bit of bands can be seen on lane 3 – 5
Do transformed. see what will happen

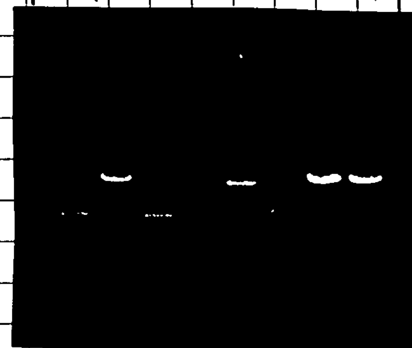
2. Pick another 4 colonies on old ligat
→ 3 ml LB/tmp

1. No colony on plate

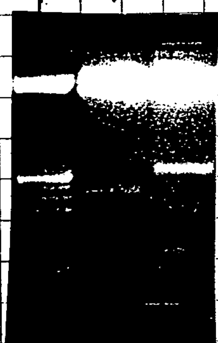
2. minip rep of plasmids by Promega kit
elute DNA in 50 ul ddH_2O

① cut with XhoI

colony 9 has insert



② cut with BglII + XhoI
BglII + XbaI



a. XhoI + BglII Bands show right vector with right insert

b. XbaI + BglII show <u>backward</u> insert

I have two options for nest steps

① get more colony 9, cut with XhoI then re-ligated. I should be able to get right insert + vector with right orientation.

② Reprepare the vector/CIP + F 8 and re-do ligation.

First I will do ②.

3. prepare the new Batch of vector and FⅧ.

① FⅧ

ddH2O	22.6 ml
10× NEBuffer 2	4.4 ml
BSA	
pBCL2-C6	10 ml (2 ug)
XhoI	3 ml.
	40 ml 37°C ~~overnight~~ 2 h

② Vector

ddH2O	14.2 ml
10× NEBuffer 2	3-3
BSA	
DCL5-C8	20 ml
XhoI	2 ml.
	40 ml 37°C 2 h

run the 0.7% agarose gel. get the bands purified through agarose gel kit

③ Dephosphorylation of vector

ddH2O	0
10× NEBuffer 3	5 ml.
DNA	40 ml.
CIP	4 ml
	50 ml. 37°C 1 h.

Add 2.5 ul 100 mM EDTA → 5 mM

75°C 10 min

purify through QiaEx II kit, elute DNA in 20 ul 10 mM Tris-Cl

4. Ligation

① DL7 5/X ho7+c2p 4 ul
ddH2O 4 ul

② F-8 4 ul

③ D - 4
F - 4.

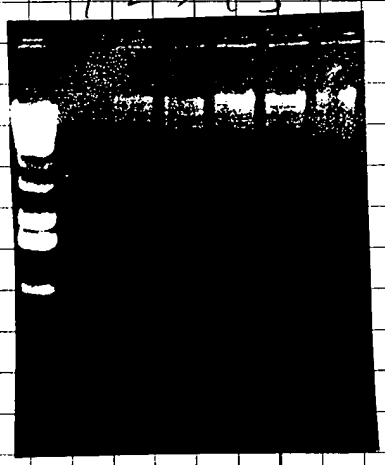
④ D - 2
F - 6.

⑤ D - 1
F - 7

⑥ D - 0.5
F - 7.5

16°C overnight

① run the ligate sol.
and. from rest of each system.
Compared to May 18's ligate
picture.



① the insert (F8) were ligated.

② the vector no obvious
change.

Do the Dephosphorylate damage
the DNA?

② Do transformat.
3. 4. 5. 6.

the nuber of colonies are
3 > 4 > 5 > 6.

pick 10 colonies from 3 & 5.
to 3 ml LB/Amp → 37°C
overnight.

① Digest pIR-EGFP/XbaI with BclI (Brand new from NEB)

dd H2O	0
10x NEBuffer 3	13 uL
DNA (P113)	112 uL
BclI	5 uL
	130 uL

50°C 2 h overnight

② D(7) – C4.2 / XbaI + BamHI

dd H2O	10.5 uL
DNA	30 uL
10x BamHI buffer	
+BSA	5.5
BamHI	2 uL
XbaI	2 uL
	50 uL

37°C 2 h

load to 0.8% agarose gel.

pIR-EGFP can not cut by BclI.

purify DNA from Gel by Qiagen gel kit. elute DNA in 100 uL 10mM Tris-Cl (pH 8.5)

③ Re-cut the pTR-eGFP by XbaI or BclI to ~~see~~ at last what happened and will happen.

ddH2O	1 ul	0.4 ul	
10 X NEBuffer 3	6 ul	6.6 ul	10 X NEBuffer 2 + BSA
DNA	48 ul	48 ul	
~~XbaI~~			
BclI	5 ul	5 ul	XbaI
	60 ul	60 ul	
	50°C	37°C	
	overnight	overnight	

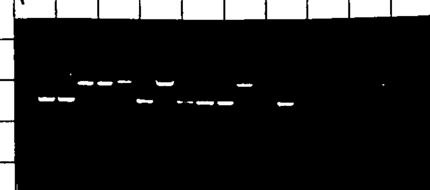
④ Extract plasmid DCZ + F8 by Promega mini prep kit. elute DNA in 50 ul ddH2O

cut with XhoI

ddH2O	14.7 ul
10 X NEBuffer 2 + BSA	3.3
DNA	10 ul
XhoI	2 ul
	30 ul

37°C 2h

NEB DNA ladder 1



3.1 — 10 5.1 — 10

Load to 0.7% agarose gel

3-~~3~~5, 5-2, 5-4, 5-9 have insert of F8.

⑧ further cut by BglII + XbaI
BamHI + XbaI.

BglII + XbaI

ddH2O	12 ul
10 ~~Buffer~~ + BSA	4 ul
DNA	20 ul
BglII	1.8 ul
XbaI	1.8 ul
	40 ul.

BamHI, + XbaI

4 ul. 10 × Buffer for BamHI, + BSA

1.8 ul BamHI,

1.8 XbaI

37°C 2 h.

Load to 2% agarose gel

① 50bp DNA ladder.
② 3-5 / BglII + XbaI
③ 5-2 / ——
④ 5-4 / ——
⑤ 5-9 / ——
⑥ 1-5 / BamHI + XbaI
⑦ 5-2 /
⑧ 5-4 /
⑨ 5-9 / ——

From

I picked 5 colonies from ligate

DC25/XhoI + F8.

Got 7 colonies with F8 insert.

5 backbones (vector) are without

EnhancI. (possible contaminats from F8/Xho with undigested DC25) with

2 with EnhancI, only one has

right orientation. It is today's

colony 13.

↑ Sobp DNA ladder

↑ C_{13} / XhoI + BglII = 250 + 80

↑ C_{13} XbaI + BglII = 90 + ~

Culture 200 ml bacteria with Colony 13

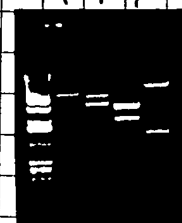
(I failed to get DNA of DL36-C13 until ... So the following were done on

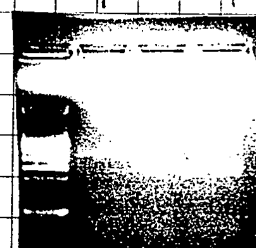
- Extract plasmid of Colony 13 by Qiagen maxi kit. elute DNA in ml TE pH8.0

Digest with.

0.7% agarose gel:
① XhoI ② 4435+3500
② BglII ③ 1945+2817+3173
③ SmaI ① linear 7815 3013+4900
④ AgeI ④ 1235+6700.

2% agarose gel:
⑤ XhoI + BglII 250+80
⑥ XbaI + BglII 180
⑦ XbaI + BamH1 190

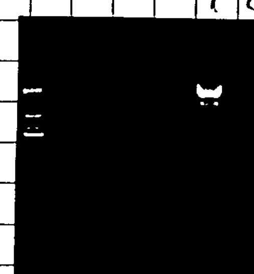




- cut PTR/uf5 & PTR-EGFP with BclI

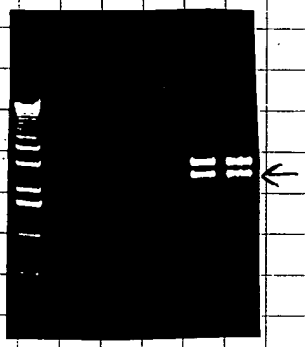
No BclI site along PTR-EGFP or uf5

I will cut PTR-EGFP with XbaI + BglII to get the EGFP + Neo cassette.



PTREGFP/XbaI	98 μl	(P123)
10 x React 3	12 μl	
BglII	10 μl	
	120 μl	37°C 2 h

Load to 0.7% agarose gel



PTR-EGFP/XbaI + BglII

⇒ 4982 (EGFP+neo)

+ 3123 (vector)

(615 CMV promoter)

Cut the bands, purify through Qiagen gel kit

elute DNA in 30 μl 10mM Tris-Cl (pH 8.0)

1. Get pCI26-CIS DNA digest (see last Page)
2. Partial digest with BglII.

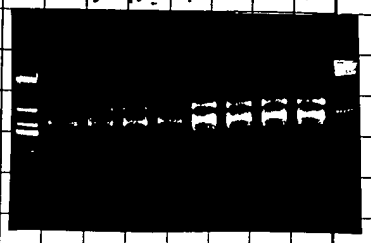
①	40 μl	DNA
	5 μl	10x NEBuffer 3
	3 μl	BseI
	2 μl	BglII
	50 μl	37°C 15' 30' 45' 60'

Add 10 uL 0.1M EDTA at every time point.

Load to 0.7% agarose gel.

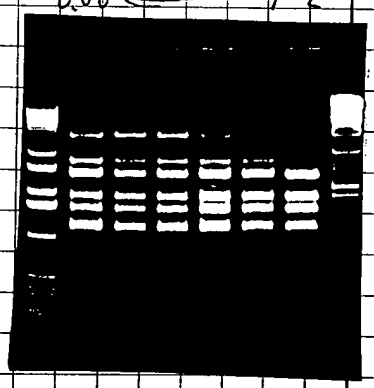
① BglII digest after xset.

② Co-digest with ISfA at simultaneously.



EDTA could not stop restrict digestion or too much enzyme or too long incubate.

3. 40 uL DNA
5 uL buffer 3
3 uL XbaI
① 2 uL BglII
② 1 uL BglII (in 2 uL)
③ 0.5
④ 0.25
⑤ 0.125
⑥ 0.06



37°C 30'

Load to 0.7% agarose gel.

Cut the bands between 4.3kb & 5kb marker.

Purify DNA by QiaEX II kit. elute DNA in 20 uL 10mM Tris.Cl

4. Ligate

F8:	①	F8 4 µl + ddH2O 4 µl	No colony
	②	PTR1/MT + CZP 4 µl + ddH2O 4 µl	—
	③	F8 4 µl Vector 4 µl	85 colonies
	④	F8 6 µl Vector 2 µl	7 colonies
	⑤	F8 7 µl Vector 1 µl	15 colonies
EGFP:	~~①~~	~~Vector~~	
	~~②~~	~~EGFP~~	
	⑥	EGFP 4 µl	200–300
	⑦	Vector 4 µl	①
	⑧	Vector 4 µl EGFP 4 µl	>300
	⑨	Vector 2 µl EGFP 6 µl	>300
	⑩	Vector 1 µl EGFP 7 µl	>300
16°C		overnight	

(1) Transformati 200 ul bacteria/dish
(2). prepare 2 liter culture of XX6. in the course, at least 1/3 were lost by carelessness. However, I sent 9.x 5 ng in total
(see last page)
for transformation

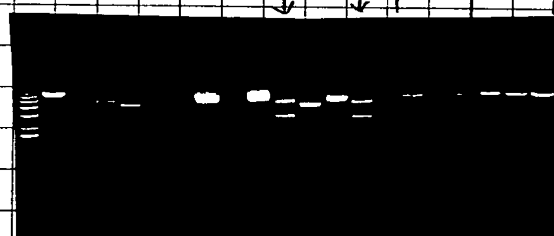
pick 14 colonies from F8 plates

pick 6 colonies from EGFP (4) plate

→ 3 ml LB/50 ug/ml Amp.

(14 from F8, 6 from EGFP)
Extract plasmids by Promega minprep kit
elute DNA in 50 ul ddH2O

C10 C13 EGFP 1-6

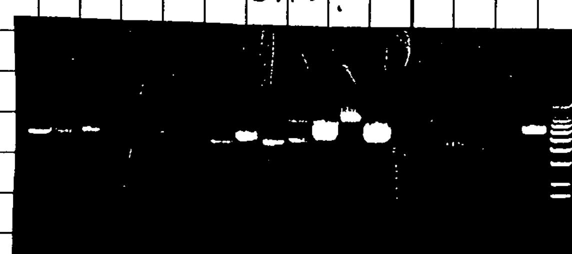


—(1)
1.5 h

for F8 plasmids (DLZ6)
cut with 2 ul (pad plasmid)
SmaI at RT / 2 h

I confused C4 & C14, however one of the two has 2 ITRS.

EGFP plasmids (DLZ8)
with XbaI + EcoRI
37°C 2 h



← (2)
2 h

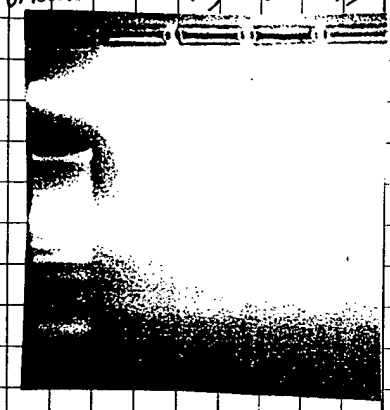
△ : (1) DLZ6 colony 10 and colony 13 definitely have two ITRS. I will do more test cuts
(2) 6 colonies of DLZ8 seem containing EGFP insert. however the enzyme failed to cut

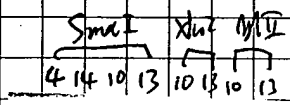
DLZ6

1. Get more plasmids of colony 10 & 13 do more test cuts

① NheI + XhoI & NheI + XbaI

vigna





② SmaI.

~~NheI~~ XhoI.

NheI

C4 has 2 NheI

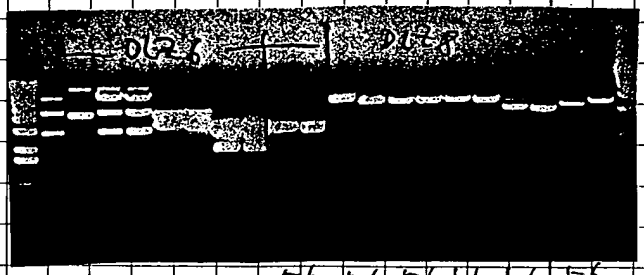
XhoI & NheI cuts of 10 & 13 are ok

Select C13 to sequence.

2. Cut DLZ8. C5 & 6 with more enzyme

the results are confusing.

I will send C6 to sequence.



① prepare LB medium to make more plasmids.

② test-cut XX2.

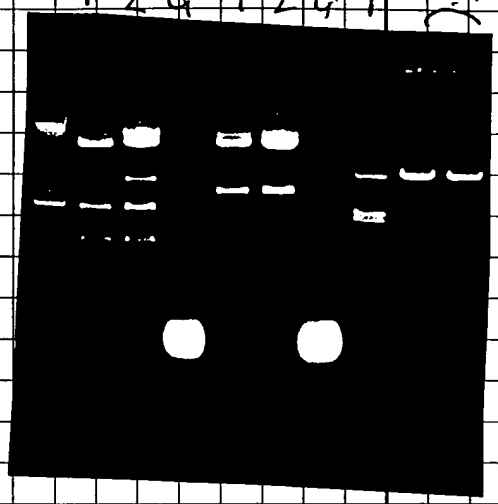
1: colony 1

2: colony 2

G: plasmids XX2 from Genbio.

from HincII & PvuI cuts

the 2 colonies have the same bands with primary plasmids.



③ Transfer EnhancII TIKT MCST EGFPtPA cassette to pTP backbone with two ZFPs.

④ prepare culture of plasmids XX2 2 liters

XX6 1 liters

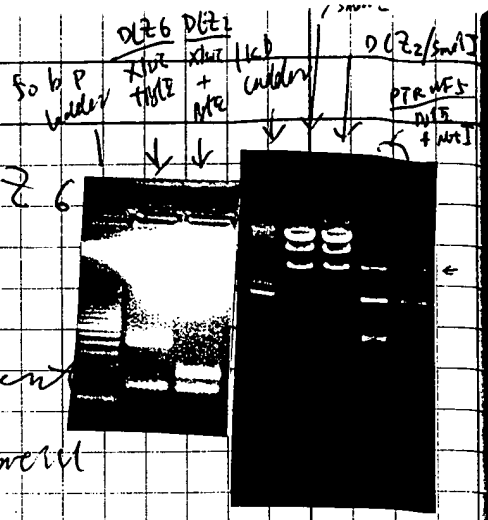
pUZ6-C13 0.5 liters

pUZ2-C3 0.5 liters

⑤ Extract plasmids by Qiagen Mega plasmids kit.

dissolve DNA in TE (pH 8.0)

⑥ plate 48 plates 293 cells (P32)



① test cut of DLZ2 & DLZ6

② 293 cells only 20-30% confluent perhaps I need to wait overnight tomorrow for transfection

293 cells : 40-50% confluency

DMEM from Sigma?

Transfection:

① change media at 6:00 pm

② start transfection at 9:00 pm

			12 x
vector	DLZ2	15 ug	180 ug (450 mL)
	DLZ6	15 ug	(600 mL)
XX2		15 ug	180 ug (150 mL)
XX6		45 ug	540 ug (250 mL)
2.5M CaCl2		125 mL	1500 μL
ddH2O			DLZ2 12.65 mL
			DLZ6 12.50 mL

7.5 mL → 2x HBSS

2.5 ml per dish. (end at 9:50)
I used vortex machine this time
the precipitate could be seen just 1~2
minutes after mixture.

feed transfected cells with warm
CO_2 – saturated DMEM/10%FBS/Pen.Strep
at 8:30pm

Harvest 293 cells at 12:00 pm.
most of plates reach 90% confluency
(60% confluency at transfect.)
some cells floated.
put the cells to −80°C.

Time 11.
Thaw – freeze at 37°–80°C 4 times.
Sonicated.
precipitate the viruses.
Set ultra-spin. 4h 15°C.

Drip the Gradient of ultra-spin

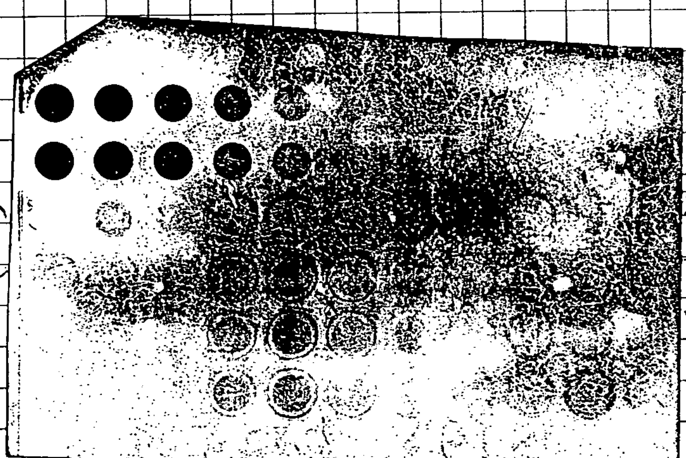
do dot-blot to titer the rAAV/E8

Duplicate Sender < A $\frac{1}{100}$ → 1/5 serial dilution
B

Duplicate 10 ul DEZ2 { C
D

DEZ6 { E
F

1. Develop the membrane



Tube 4 5

	plasmid	
①	100ng	2.35×10^{10}
②	20ng	4.2×10^{9}
③	4ng	9.4×10^{8}
④	0.8ng	1.88×10^{8}
⑤	0.16	3.76×10^{8}
⑥	0.032	7.52×10^{6}

$\frac{1.88 \times 10^{8}}{10\ ul} \times 1500\ ul$

$= 2.82 \times 10^{10}$

• pour 4 & 5 together. dialyze against rAAV-Dialysis-Buffer overnight

2. plate 8 × 10^5 /well & 1 × 10^6 /well

HepG2 cells (P_{10}) in 6-well plate

Monday

① prepare media for F8 cell culture

a. I screen the F8 in FBS last Friday by APTT

UCRD: 1:40 26.4
1:80 91.4
1:160 103.9
1:320 104.9
1:640 142.4

Serum: Tube 1 2 3
149.9/142.4 135.9/146.K 102.4/108.9

At least I can use serum in tube 1 and 2

b. ~~FBS~~ 10% FBS + Penicillin/streptomycin + 20ug/ml aprotinin (Sigma)

500 ml ~~DMEM~~ Minimal Essential
60 ml FBS
600 ul 100x P/S

100 ml 10% FBS/DMEM + PS → 20 ug/ml
2 ml 1mg/ml aprotinin

C. infect the HepG2 cells

I will use 1×10^6/well cells

2×10^5 seem a little bit less. and the HepG2 cells grow slower than 293 cells, There should be 5 days for them to confluency

around 3:00pm

① wash the wells with DMEM

② Add the viruses ① ② ③ ④ ⑤ ⑥

DLZ2 : 1ml → 2

DLZ6 : 1ml → 5

37°C for 2h swirl plates every 15'

③ suck off ~~media~~ viruses
add 2ml of MEME to each well
10% FBS, P80
Aprotinin

△ The ~~construct~~ DLZ8 → EGFP + Neo ~~insert~~

Worked bad

I could get the expected band by BglII cut

but not from HindIII. XbaI. XhoI. etc. cuts

sequence failed.

I donot know the reason | On the map of BM Caverns, because there is another BamHI site behind polyA of P(Z) so the 3' part of MCS and the entire polyA are deleted. Revin

~~from last Tuesday — Friday~~

I prepared more pTREGFP plasmids.
transform ~~screen~~ the bacteria.
pick colonies (4)
overnight culture — mini — prep.

① the plasmids do not have DcII site!

② I cut the EGFP + Neo + poly(A) by XbaI + HgII

③ sequencing of the DLZ8 — C4 & C2 show the right insert of MCS. I cut C4-2 with XbaI + BamH1.

④ ligation

4 ul ddH2O { ① DLZ8 4 ul.
② pTREGFP colony 4 4 ul.
③ ① + ②

16°C overnight.

Transform DH5α competent cells this afternoon. 100 ul bacteria / dish.

Tuesday.

1. transformation of DLZ8

No colony on plate ①.
about 20 colonies on plate 2 → contamination from pTREGFP only once cut by XbaI or HgII?
> 200 on plate 3
pick 8 colonies from plate 3 overnight culture

2. feed HepG2 / F8-VMU cells with

DMEM + 10% FBO FBS + 20ug/ml aprotinin

store the changed media at -80°C

3. count the cells at 3:00pm

There are still 1×10^6 HepG2 cells / per well

24 hours later

48h. 3:00pm) : 3.2×10^6.

2×10^5 HepG2 cells. 48h later. 3.8×10^5

Extract the plasmids with Promega wizard mini-kit

(cut with XbaI + BglII = 381 + 2493 + 3123)

first. I cut the plasmids with XbaI + HindIII and expected a 2.7kb + a 3.3kb bands

actually, I only could get a 6.0kb bands.

which mean only XbaI or HindIII site along this plasmid. just as I got before.

1 —— 8 / XbaI + HindIII

1kb DNA ladder

I Reviews the maps of pCZx-Cx and PTR-EGFP found there is another BamH, site between poly A and ITR. that means the 3' part of mcs at the poly(A) in pCZx-Cx were deleted. So That is the version.

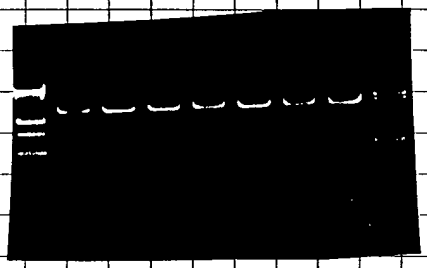
Recut the plasmids pCZ8 colony 1~8 but 2. with XhoI + HindIII

PTR-EGFP / XhoI + HindIII

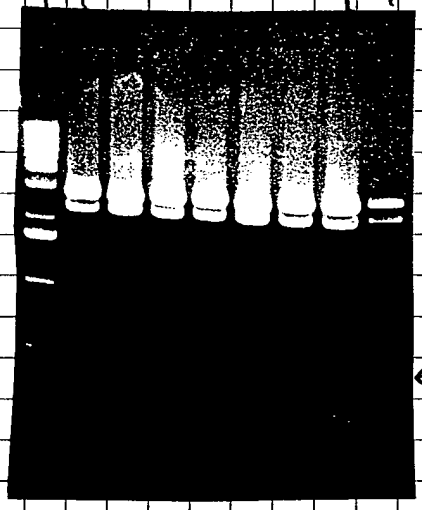
1kb ladder

pCZ8

PTREGFP



1 th



2-5 th

from pCZ8 a 3200bp band means EcoRI + 5' MCS

PTREGFP a 720bp ——— CMV promoter

Confirm the insert of EGFP to vector pCZx-Cx

① Aptt of the supernatant of HepG2/F8

UCRP. 200 ng × 103% F8 = 206 ng/ml

1:10	100% activity.	
1:20	50%	
1:40	25%	
1:80	12.5%	91.4 seconds
1:160	6.25%	102.4/105.9/102.0/99.4
1:320	3.12%.	122.4/118.9/119.4
1:640	1.6%	138.9/138.9/138.9

post-infection	1×10^6 HepG2/None	BAU/DLZ2 2×10^4/cell	BAU/DLZ6 2×10^4/cell
Day 1	142.9	—	132.4/144.9
2	—	—	112.9/123.9
3	144.6/141.9	139.0/142.3	99.4/103.9/108.9
4	143.4/139.4	129.4/132.4	113.9/111.9/103.9/112.9

I can see 3~6% activity of F8 in the overlay of cells. remain to be confirmed by Elisa & Coatest Assays.

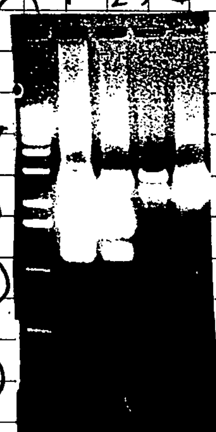
tranfer HepG2 cells to 10-cm-dish in full medium.

② Digest pLZ8 (without 2 DTRs) with

a. BglII to get EnhII + TK + EGFP + Neo + PolyA cassette.

b. XbaI + BglII to confirm EnhII + TK replacement of CMV promoter in pTR-EGFP plasmid

1% agarose gel.



(1) (2) pLZ8 / AseI + BglII (~~+2487+3123~~ 184 + 1235 + 1254 + 2864)

3 pTR-EGFP / BglII + XbaI (615 + 2487 + 3123)

4 pLZ8 / BglII + XbaI (321 + 2493 + 423)

③ Cut the bands, elute DNA in band positions.

④ Ligate:

① pTR / BglII + CIP 4ul + 4ul ddH2O

② EnhII + TK + EGFP + Neo + Poly / BglII 4ul + 4ul ddH2O.

③ Vector + insert
4ul 4ul.

1ul T4 ligase + 1ul 10x ligase buffer

16°C, overnight

Add 10 ul TE (pH8.0) to the ligati soln. use 10 ul to transform DH5α cells. 100 ul/dish
Number 4 dish is paul's hufⅨ rAAV plasmid.

① Transformati:

plate# ①. No colony

②. < 10 colonies. ②.

③. > 200 colonies

④ > 500 colonies.

pick 8 colonies from plate# 3

4 4

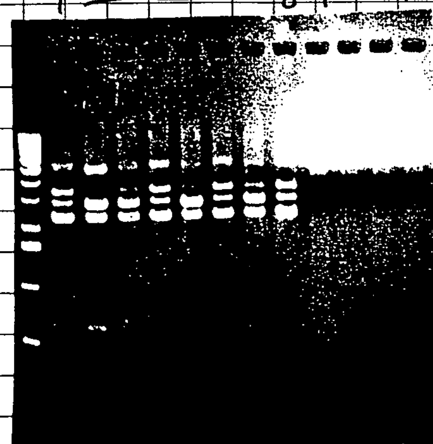
→ 3 ml LB/25 ug/ml LB. 37°C overnight.

② HepG2 cells/pZZ6 died. No reasons known.
I freeze the cells to see whether I can get some RNA for Northern blot

Extract the plasmids by promega's miniprep kit
elute DNA in 50 ul ddH2O
use 10 ul of each to Digest with SmaI

for DLZ8
both ITRS < 638bp, 2264bp, 2945bp
have 5' ITR < 638 bp, 5370 bp
3' ITR < 2264, 3723

DLZ8 1 — 8, hufIX 1 — 4



1kb ladder

So the colony 3, 5, 7 have more intact 2 ITRS

for hufIX plasmids, only one ITR there. I will pick another 8 colonies to screen ITRS. if there still no colony with 2 ITRS, I need talk with chris.

use DLZ8 — C5 to make more plasmids

B/25 ug/ml Amp 37°C overnight.

8. pTRUF5 by Qiagen mega kit
ml TE (pH8.0)

DNA/Oligo Quant

ReadSamples RawData Method SaveClear Print Quit

Results file: A:\WORK_RES Method name: A:\DSDNA

Assay type: ds DNA
Sampling device: None
Read average time: 0.50 sec

Background corr: [Yes] 320.0nm
Pathlength: 1.0000 cm
Conc. factor: 50.000 at 260.0nm

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/280.0	Dil. Fact.	Conc. ug/mL
PTRUF5	0.1562	0.0857	1.8231	150.00	1171.4393
DLZ8	0.2561	0.1337	1.9152	150.00	1920.9821

① try to prepare more xx6 and DLZ6 plasmids by CsCl2 gradient methods

However, the yield of plasmids were low.

From 1 liter media only get 0.5 mg plasmids.

BECKMAN DU 640

Time: 11:46

DNA/Oligo Quant
ReadSamples RawData Method SaveClear Print Quit

Results file: A:\WORK_RES Method name: A:\DSDNA

Assay type: ds DNA
Sampling device: None
Read average time: 0.50 sec

Background corr: [Yes] 320.0nm
Pathlength: 1.0000 cm
Conc. factor: 50.000 at 260.0nm

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/ 280.0	Dil. Fact.	Conc. ug/mL	
DLZ6	0.0685	0.0359	1.9112	150.00	514.0432	x2ml
XX6	0.0274	0.0124	2.2095	150.00	205.4807	x2ml

② Tried 3 times to plate some Hela cells to test HAV-3 Ab in two dog's plasma, the Hela cells died. no Reasons known. meanwhile the 293 cells died when plated in six-well plates or 12-well plates but alive in 15-cm dish.

medin: Gibco/BRL DMEM-H, +10% FBS. with or without Ampicillins.

① plate 50 plate ~~of~~ 293 cells (~~grow~~ P30, from vector), cells grew well, but slower than usual. when plated the cells in 6-well plates, died. no reasons known. I have frozen 20 vials of cells in liquid nitrogen. ~~I wonder the viability of them. Now.~~

② Transfection.

30 plates of pCZ8. LEGFP with Ehi(+TK)

		1X	30X	
Sol. I	Vector (pCZ6)	15 ug	450 ug	232 ul
	XX2	5 ug	150 ug	116 ul.
	XX6	30 ug	900 ug	346 ul
	2.5M CaCl2	0.125 ml		3.75 ml
+	ddH2O			33.051 ml
				37.5 ml.

Sol I 20 plates of pCZ6 — kat 8.

	20X	
vector	300 ug	584 ul
XX2	100 ug	22 ul.
XX6	600 ug	230 ul
2.5M CaCl2		2.5 ml
ddH2O		21.6[illegible] ml

2.5 ml sol'n I (1.25 ml for one plate) mix with 2.5 ml 2× HeBS, when the precipitates were seen, add 2.5 ml to each plates

③ Feed the transfected cells with pre-warmed and CO_2-saturated media/10% FBS on the next day of transfect. ( )

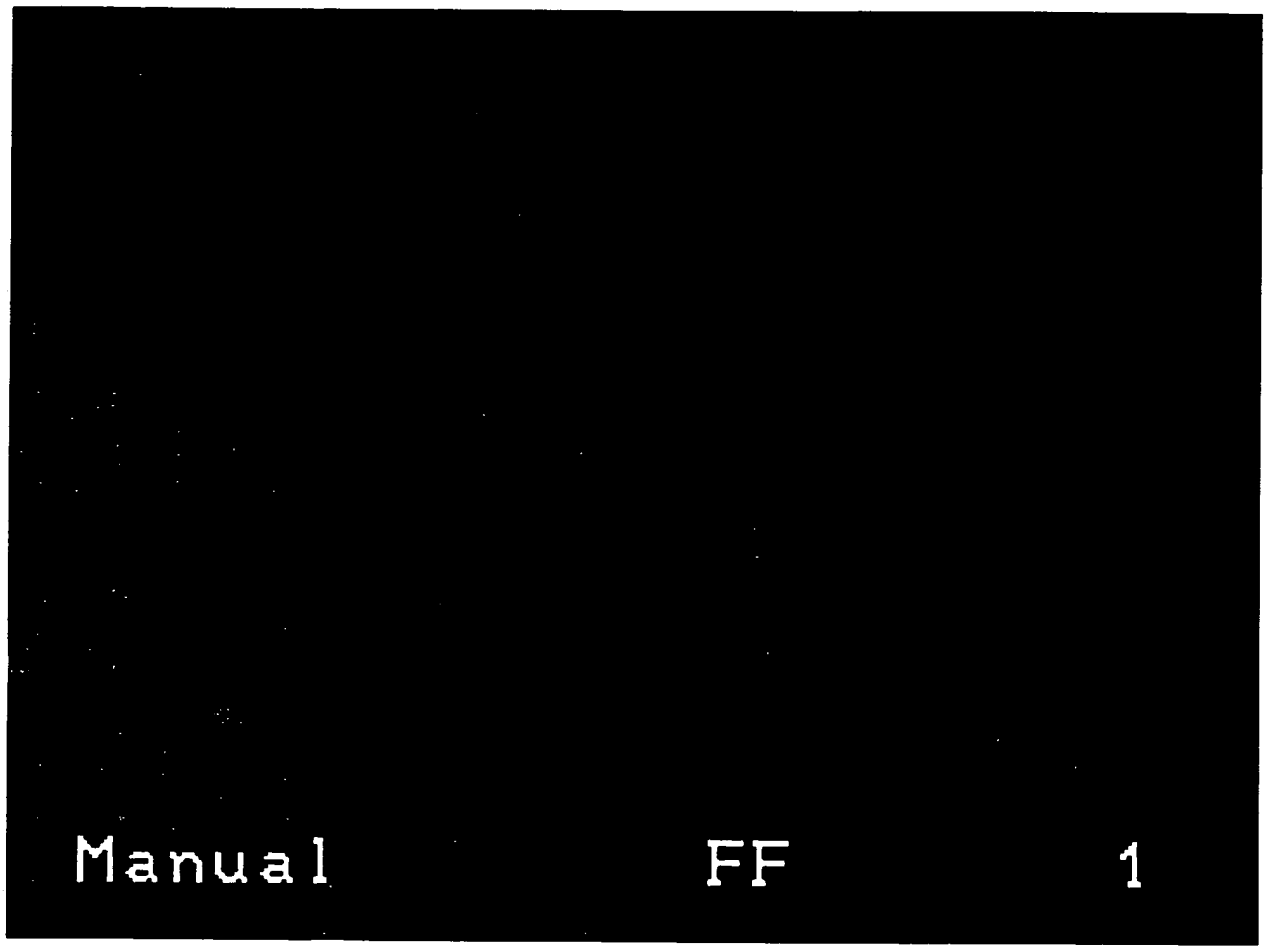
the EGFP cells (green cells) did not appear 8 hours post-transfect.

very significant 20 h post-transfect.

from percentage of green cells in transfected dishes, the transfect efficiency are around 60-70%, similar to the former.

⑩ Get Rat liver cell lines from Dr. Coleman's Lab (Brinkhous-Mulock RLG) using Richter I-10/10% FBS (Gibco/BRL)

Transfected 293 cells with DCZ.R

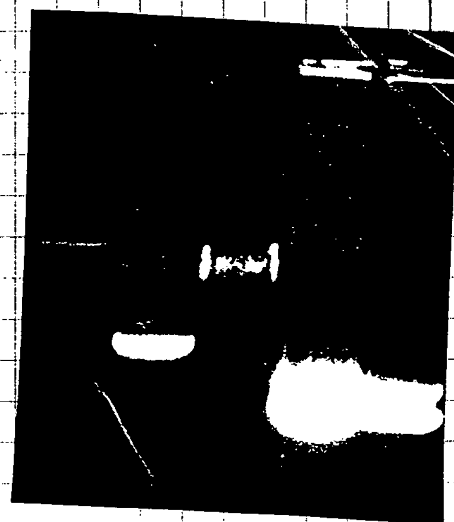


① harvest the making AAV 293 cells release the viruses and set ultra-spin 60K. 41K. 15°C

② get EGFP template for probe labelling

cut the Bands purify them Qiagen gel kit. elute DNA in 100ul 10mM Tris/(pH8.0)

EG



← EGFP +Neo.

pCZ8/XbaI+BglII

① label the EGFP with Dig following routine protocol

② drip the CsCl2 gradient of rAAV/EGFP & F8.

set dot-blot. 10ul of each well.

1 2 3 4 5 6 7 8. 9. 10 11. 12

plasmid pCZ8 standard A
AAV EGFP/tube 1 B
2 C
pCZ8 standards D.

plasmids pCZ6 standard { E F

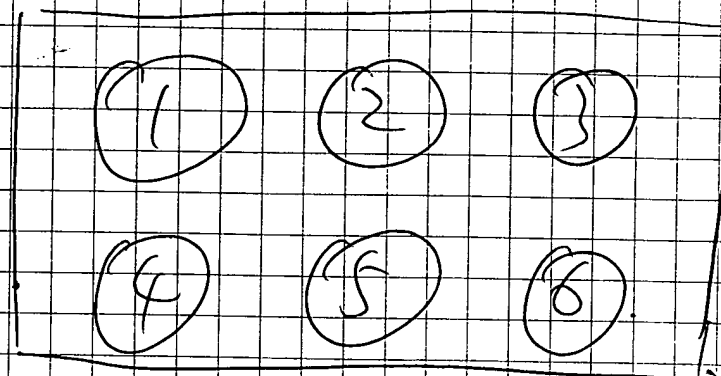
AAV/F8 tube 1 G

tube 2 H

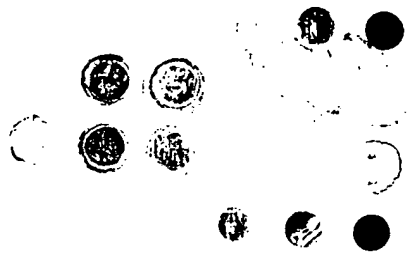
① develop color of dye-boil

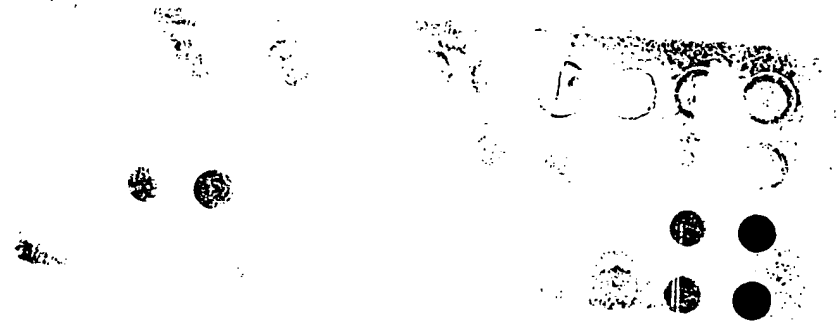
② Dialysis RPMI. < DLZ6: 2 tube 4.
DLZ8: 2 tube 5.
change dialysis buffer after 6 hs.

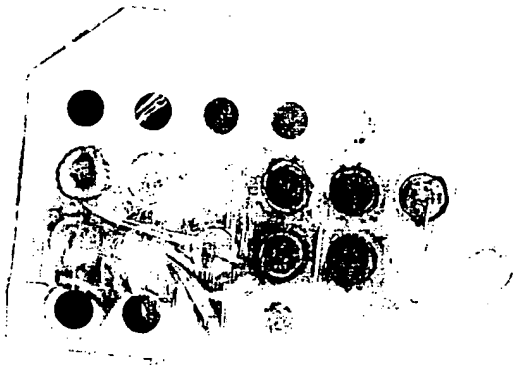
③ plate cells for activity-assay of hu-IFβ.

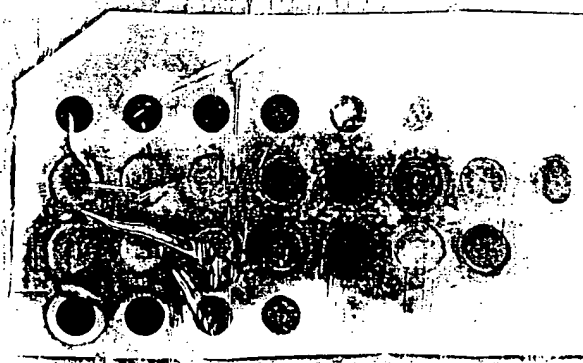


293 cell : 10% FBS+ DMEM.
HepG2 : 10% FBS+ MEM&.
WISH-3AA : 10% FBS+ Richter 1-10 (Gibco/BRL)



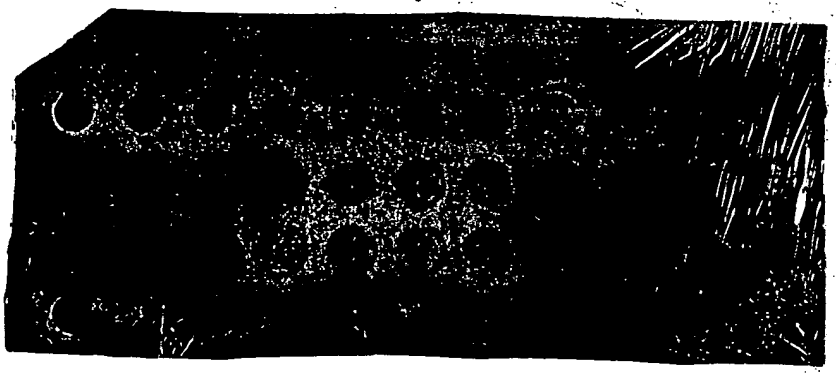






↑ DLZ6

↓ DLZ8



④ functional titer of rAAV/DG28 (GFP)

a. physical titer of rAAV/p8

$= \frac{2 \times 10^9}{10\,ml} \times 3\,ml = 6 \times 10^{11}$ particles

b. rAAV/GFP $= \frac{3.5 \times 10^9}{10\,ml} \times 6.25\,ml = 2.36 \times 10^{12}$

	DG26	DG28
100 ng	2.35×10^{10}	8.1×10^{10}
20 ng	4.7×10^9	6.2×10^9
4 ng	9.4×10^8	1.24×10^9
0.8 ng	1.88×10^8	2.48×10^8
0.16 ng	3.76×10^7	4.96×10^7

c. 1×10^5/well Hela cells in 12-well plate

12 hours post-plating, Ad MOI = 10 1 h

well ① 10 ml rAAV/GFP + 100 ml DMEM 10 ml

well ② 10 ml ① + ——— 1 ml

well ③ 10 ml ② + ——— 0.1 ml

well ④ 10 ml ③ + ——— 0.01 ml

20-30 green cells in each field

1-2 green cells in some field

No. green cells

infect cells with rAAV/hF8 (DL7G)

the viruses I used is from tube 4.

the titer is $\frac{2 \times 10^9}{10\,ml} \doteq 2 \times 10^8/ml$.

so. for the 293 cells (2×10^5) : 100 ul
+ 900 ul DMEM

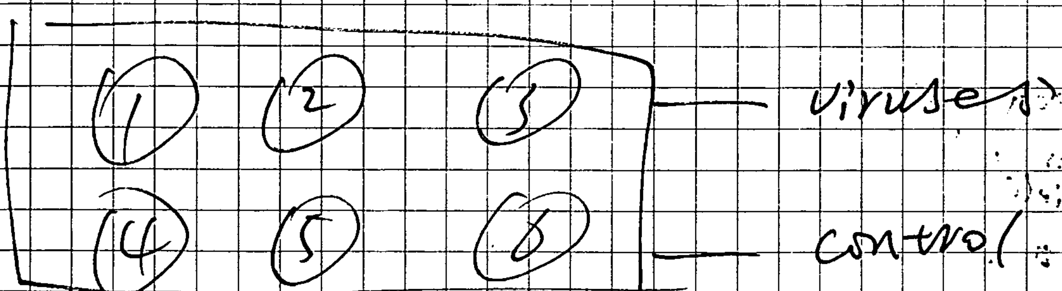
HepG2
WBF344) (4×10^5) 200 ul
+800 ul DMEM.

① wash ~~plate~~ each well with 2 ml DMEM.

② Add the viruses, incubate at 37°C for 1 h. rock the plate every 15 min.

③ suck off the viruses, wash the well with DMEM.

④ Add 2 ml relevant media/FBS to each well. (at 12:00 finish infection)



Remark: ① 293 cells, most were detached, and some were lost during the course.

② 4×10^5/well WB F344 are too heavy confluency, they are nearly 100% confluency while infection.